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The Patent Office

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1. Your reference

PP/2548

2. Patent application number
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9606040.5

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

ISIS INNOVATION LIMITED
2 South Parks Road
OXFORD
OX1 3UB

Patents ADP number *(if you know it)*

3997856001

If the applicant is a corporate body, give the country/state of its incorporation

UNITED KINGDOM

4. Title of the invention

ACTIVE PEPTIDE

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

STEVENS HEWLETT & PERKINS
1 Serjeants' Inn
Fleet Street
LONDON
EC4Y 1LL

Patents ADP number *(if you know it)*

1545003

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

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Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
See note (d))

Yes

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Continuation sheets of this form	
Description	12
Claim(s)	1
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Priority documents	
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Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	

11. I/We request the grant of a patent on the basis of this application.

Signature Stevens, Hewlett & Perkins Date 22-03-96
Agents for the Applicant

12. Name and daytime telephone number of person to contact in the United Kingdom: P Pennant; 0171-936-2499

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ACTIVE PEPTIDE

This invention concerns the enzyme acetylcholinesterase (AChE) in which the inventors have identified a biologically active peptide.

The classical or cholinergic role of AChE is to degrade enzymatically levels of extracellular acetylcholine. However, it has long been known that AChE exists also in a soluble form, (not a requirement for its classic enzymatic role) and is found in parts of the body where there is little or no acetylcholline. It is becoming widely accepted that AChE has a non-cholinergic function, though the biochemical basis for this function remains unclear.

It is believed that excessive AChE may enhance calcium entry into cells independent of its normal enzymatic action. Elevated cellular calcium levels may lead to a range of pernicious consequences, including undesirable changes in gene expression and, more importantly, mitochondrial swelling which may thereby compromise ATP metabolism and may indeed lead to apoptosis or programmed cell-death. Disease states which may be implicated include Parkinson's disease, Alzheimer's disease, stroke and malignancy.

The cytoplasm of cells typically contains calcium at concentrations of the order of 1 μm. Calcium is present intracellularly in the endoplasmic reticulum in millimolar concentrations. Extracellular body fluids contain calcium also in millimolar concentrations. A calcium pump operates to maintain this substantial concentration difference between the cytoplasm and the endoplasmic reticulum, and thapsigargin is known to be implicated in the breakdown of this pump. Similarly a calcium pump normally functions between the cytoplasm and the extracellular fluid. It is believed that the consequences of the action of excessive AChE may be

comparable to the breakdown of these pumps.

AChE, acting in a non-cholinergic capacity, has been shown to play an important part in the normal and abnormal functioning of the substantia nigra, the region affected in Parkinson's disease. There are are three possible ways in which AChE may have toxic effects:

(i) excessive AChE may be released as a consequence of compensatory mechanisms known to occur in that disorder;

(ii) excessive glutamatergic activity known to occur in Parkinson's disease may lead to over-stimulation of calcium channel N-methyl-D-aspartate (NMDA) glutamate receptors, thereby converting a physiological situation to a pathological one;

(iii) normal levels of AChE may act synergistically with fragments of β-amyloid precursor proteins known to be present in the Parkinsonian substantia nigra.

AChE, again acting in a non-cholinergic capacity, may be an important contributing factor in Alzheimer's disease. In transgenic mice with excessive AChE there are cognitive deficits reminiscent of Alzheimer's disease. Moreover Alzheimer's disease has been directly associated with inappropriate levels and forms of AChE. Excessive AChE may act to enhance calcium entry through overactivation of otherwise normal adaptive processes via a mechanism discussed in the experimental section below.

Current therapies for both degenerative diseases are somewhat inadequate. Anti-Parkinsonian drugs which target dopamine substitution do not arrest neuronal cell loss, and newer drugs aiming to block calcium entry directly may have poor net payoff in terms of neuronal health and in addition would have widespread undesirable effects in both the central nervous system and peripheral tissue. Moreover, drugs used in Alzheimer's disease which exclusively target the cholinergic system, neglect areas where AChE may be having its pivotal non-cholinergic function. Previous attempts to target calcium channel activity in therapy for

neurodegenerative disorders have been hampered by the non-selective effects of the compounds available.

In order to be conveniently administered, a compound for treatment of disorders of the central nervous system, or more particularly of the brain, needs to be capable of crossing the blood-brain barrier. AChE is not capable of doing this, though a small lipid-soluble analogue might be. Workers in the field have been seeking biologically active peptides based on the AChE molecule for more than ten years, in the hope of thereby achieving a more effective and selective treatment for disorders of the central nervous system such as Alzheimer's and Parkinson's diseases.

It is known that antagonism of NMDA receptors is being explored as a therapy for stroke. The present invention is expected to find application in specific therapies for combating stroke and other problems of cerebral circulation.

Abnormal cholinesterase expression occurs in several types of tumour cells. Although the role of cholinesterases in tumorigenesis is unclear, the fact that AChE and BuChE (butyryl cholinesterase) may be involved in the control of cell growth and proliferation during early development suggests that the amplification of cholinesterase genes may influence the ability of tumour cells to proliferate more rapidly. According to the invention, antagonists of the non-cholinergic action of AChE are expected to be of interest in the prophylaxis and treatment of cancer.

Amyloid precursor protein (APP) is known to have similar features to AChE as follows. Both AChE and APP are secreted from neurons into the cerebro spinal fluid (CSF), where for both AChE and APP there is a decrease in CSF levels in Alzheimer's disease. Both AChE and APP can have trophic functions.

Both AChE and β-amyloid enhance calcium entry through NMDA receptors. Both AChE and APP activate potassium channels, probably linked to changes in intracellular calcium. Both AChE and

β-amyloid activate macrophages. Low stimulation of NMDA receptors has trophic effects whereas high stimulation is toxic. The dual trophic-toxic action of both APP and AChE may thus be mediated via NMDA receptors. A similar dual action via NMDA receptors has already been shown for the trophic factor BDNF in cortical cells. Finally, β-amyloid and the monomer of AChE can bind together as a complex.

This invention results from the inventors' identification of a region of the AChE molecule from which a biologically active peptide (obtained either synthetically or by endogenous processing) can be derived. The peptide consists of 14 residues of AChE from residue 586 to residue 598 (in the translation of the mRNA sequence, EMBL accession hsache. empri, number M55040, beginning at nucleotide 157). The sequence of this peptide is amino Ala - Glu - Phe - His - Arg - Trp - Ser - Ser - Tyr - Met - Val - His - Trp - Lys -carboxy, or in the one letter code, AEFHRPSSYMVHWK. The inventors propose that this, or a related, peptide from this region of AChE acts alone or in synergism with a fragment of beta-amyloid to contribute to neuronal degeneration. The invention thus provides in one aspect a peptide containing at least six amino acid residues and having at least 70% homology with part or all of the above sequence. Preferably the peptide contains at least 12 amino acid residues having at least 90% homology with the above sequence.

It appears that the two amino acid residues -Val -His -, appearing at positions 11 and 12 in the above sequence, may be of critical importance. Thus the invention also envisages peptides comprising or consisting of the four-mer sequence YMVH or MVHW or VHWK and having at least 70% homology with part or all of the above AChE sequence.

A somewhat similar peptide is present in a region of the β-amyloid precursor polypeptide. This region lies at the amino terminus of the 42 residue peptide that accumulates in Alzheimer's disease and has

the sequence amino - Asp - Ala - Glu - Phe - Arg - His - Asp - Ser - Gly - Tyr - Glu - Val - His - His - Gln - Lys - carboxy, or in the one letter code, DAEFRHDSGYEVHHQK, corresponding to residues 597 - 612 of the translation of the human amyloid A4 precursor polypeptide (EMBL accession hsafpa4. empri, number Y00264, beginning at nucleotide 148).

The accompanying table shows the multiple sequence alignment of 5 AChE sequences, three BuChE sequences and the human amyloid precursor polypeptide at the region of interest. As reported in the experimental section below, the human amyloid precursor fragment does not itself exert calcium channel opening activity, but it does enhance the activity of the AChE fragment. The BuChE fragment appears inactive, both alone and together with the AChE fragment. In another aspect the invention thus envisages a mixture of AChE peptide with another peptide having at least four amino acid residues preferably including VH and having at least 70% homology with the above β-amyloid precursor sequence.

There are various ways in which this AChE peptide or this peptide mixture may be used:-

a) Since the AChE peptide is shown to have nanomolar affinity for a binding site in the vulnerable cells, the peptide (or mixture) can be labelled with a signal moiety, or alternatively immobilised, and used to locate and identify the receptor site of the cells. The nature of this signal moiety is not material, and the technique of labelling peptides with signal moieties is well known. The peptide (or mixture) can be used as an affinity ligand for the selective retrieval of the receptor molecule itself from preparations derived from those vulnerable cells. Additionally, the peptide affinity ligand could be used to screen an appropriate cDNA expression library to isolate a cDNA encoding the binding site directly. Once that receptor site is known, it will be possible to modify or control its properties.

b) An alternative and preferred approach is to find a substance that inhibits the action of the biologically active peptide or mixture. For example an antibody or other substance which binds to the peptide would be expected to inhibit its biological action. Structural properties of the active peptide itself, together with a combinatorial analysis of the optimal peptide sequence for biological activity, will provide additional information. This structural information may suggest a family of synthetic (non-peptide) compounds that could rationally be tested for efficacy.

In a further aspect, the invention thus envisages a compound which inhibits a biological activity of the AChE peptide or peptide mixture described above. The biological activity is expected to be a calcium-channel-opening activity. The compound will preferably be capable of crossing the blood-brain barrier.

Thus the non-cholinergic action of AChE, as mimicked by its 14 residue peptide, may be selectively blocked by a synthetic compound devised in this way. Moreover, the process of developing such a synthetic inhibitor is simplified by the demonstration of biological activity in such a small sub-fragment of the AChE molecule. A consequence should be that the synthetic compound offers a more physiological action, thus reduction of calcium entry into vulnerable cells rather than complete abolition. In addition, this action should occur selectively, only in locations within the brain where AChE has a non-cholinergic action. It should be noted that these are the very sites primarily affected by cell loss in Alzheimer's and Parkinson's diseases. Thus use of these synthetic compounds should avoid widespread disruption of cellular calcium regulation, by offering a highly region-selective action within the brain.

EXAMPLE 1

Strategy for the identification of a receptor for the AChE peptide.

1. Use the peptide, tagged with biotin, to search for a cell type with a high affinity binding site for the peptide. Note that this search will begin with neuronal-derived tissue culture cell lines that should be good candidates. Functional significance for the binding of the AChE peptide will be assessed by looking for physiological effects of peptide binding, such as transient calcium currents.

2. Having identified a cell type with a high affinity binding site for the peptide, the receptor will be identified by ligand overlay blotting and intracellular localisation by indirect detection of the biotinylated peptide using a streptavidin conjugated fluorochrome. Subsequently the receptor will be purified either by affinity chromatography using immobilised peptide, or by conventional column chromatography using the ability of the peptide to bind to column fractions as an assay to follow purification.

3. The purified receptor will be subject to N-terminal microsequencing (or tryptic fragments will be purified by HPLC and microsequenced if the receptor molecule as isolated proves to be N-terminally blocked). The peptide sequences obtained in this way will be compared with a non-redundant compilation of available peptide sequence databases to identify any similarities (or identity with a known surface molecule). The sequences will also be compared with expressed sequence tag (EST) databases in case the mRNA for the receptor has already been obtained as a cDNA by chance in a random library construction and sequencing project.

4. If the strategy in (3) does not identify a cDNA sequence, the peptide sequences will be back-translated to provide nucleotide sequences from which oligonucleotides will be constructed. These oligonucleotides will be used to amplify regions of the parent mRNA by reverse transcription

of total cellular RNA (from the cell type used in the original biochemical isolation), followed by specific amplification with each possible primer pair using the polymerase chain reaction (PCR). These PCR products will be directly sequenced by cycle sequencing using an Applied Biosystems automated sequencer.

5. The sequences obtained from these PCR products will be compared with sequences in existing nucleotide databases as in. (3). If this comparison reveals that an identical sequence has previously been obtained, then a strong candidate for the receptor gene is available (Note that this does not imply that the sequence that has been obtained previously has already been implicated in any way in the functions that the invention ascribes to this molecule).

6. If no identical or highly similar sequences are identified in (5), then the PCR-derived nucleotide fragments will be used as radiolabelled probes to screen a cDNA library constructed by oligo-dT primed reverse transcription from the mRNA of the cell type used in the original biochemical isolation. This will identify candidate cDNA clones which will then be sequenced as above. The identity of the candidate cDNAs with the protein of interest will be confirmed initially by demonstrating that the clone contains the sequences of the other PCR-derived nucleotide fragments. If an incomplete cDNA clone is obtained then 5' extension will be carried out using the RACE technique (Rapid amplification of cDNA ends).

7. The function of the protein encoded by the cDNA will be confirmed by expression of the full-length protein using a transient eukaryotic expression vector in cells previously shown not to have a high affinity binding site for the AChE peptide. Expression of the protein in these cells should result in the appearance of a high affinity binding site for the AChE peptide on these transfected cells. This will confirm that the correct sequence has been identified.

8. The cDNA sequence will be used to express the protein in a baculovirus-infected insect cell system in order to obtain large amounts of pure protein for structural studies. Note that this may require the construction of a soluble ectodomain fragment if the protein is a transmembrane molecule. The structural studies will include circular dichroism (CD) measurements, 2-D nuclear magnetic resonance analysis (NMR) and attempts at crystallisation. Pure receptor protein will also facilitate detailed analysis of the binding of peptides, or candidate non-peptide agonists (or antagonists), to the receptor using surface plasmon resonance methods.

Once the AChE ligand and its receptor are known, there are various ways of controlling or preventing their action:-

a) Destroy the ligand, i.e. identify a protease that cleaves the active peptide ligand into an inactive form and promote its activity e.g. by inducing it or impregnating it.

b) Prevent production of the ligand, i.e. identify a protease that produces the ligand and inhibit that.

c) Sequester the ligand and remove it with antibody or with soluble receptor ectodomain. A modification of this approach is possible if the AChE/β-amyloid synergy results from competition for a high affinity sequestration site different from that which produces the biological effect. Introduction of excess of high affinity site will reduce the biological effect.

d) Block the receptor with an antagonist, e.g. design an analogue that binds the receptor, competes with endogenous peptide, but does not raise calcium levels. In an experimental system showing binding and calcium ion signals, it will be possible to assay for a class of compounds that bind the receptor, compete with the peptide ligand, but do not themselves activate the receptor.

e) Uncouple the receptor from the cellular response, e.g. by preventing ligand binding to the receptor from causing cellular response by

blocking a second messenger that is preferably unique to the system.

EXAMPLE 2

The 14-mer AChE peptide, the corresponding 14-mer human BuChE peptide (AGFHRWNNYMMDWK) and the 16-mer β-amyloid peptide were synthesised and used in studies to assess their biological activity, with the following summarised results:-

(i) Electrophysiological studies

These experiments are performed on slices of guinea-pig midbrain maintained *in vitro*. Intracellular recordings are made from a rostral population of neurons in the substantia nigra under current clamp conditions. All results listed below are obtained in the presence of the sodium channel blocker tetrodotoxin. In order to facilitate visualisation of calcium-mediated potentials triggered by activation of NMDA receptors, all experiments are performed in magnesium-free perfusate. Under these conditions, results to date indicate:

(a) In 11 neurons, in concentrations ranging from 10^{-7}M to 10^{-6}M, the peptide fragment derived from AChE has a selective and reversible action reminiscent of the actions of AChE itself, i.e. with lower doses/less sensitive situations there is an enhanced calcium influx. This effect is followed by, in sustained applications/stronger doses/more sensitive neurons, a marked reduction in the calcium potentials.

(b) Under conditions where AChE is normally effective and under magnesium-free conditions, the comparable BuChE fragment appears without corresponding effect (n=3), and the analogous fragment of β-amyloid also appears ineffective (n=4). However, a synergism between the peptide derived from AChE and this fragment of β-amyloid is reflected in a reduction in the evoked calcium potential (n=7) followed by the

generation of large spontaneous thapsigargin-sensitive calcium currents oscillating in a biphasic manner (n=3).

(c) In 6 neurons, application of NMDA, which on its own produces a 'physiological' depolarisation, results, under identical conditions, in severe metabolic stress of the cell after treatment with the AChE peptide at concentrations as low as 10^{-7}M, or at an even lower concentration when combined with the amyloid peptide.

These results suggest that the peptide specified in the invention is enhancing calcium entry into a population of neurons in the substantia nigra. Once large amounts of calcium have entered the neuron, buffering mechanisms come into play, reflected by the marked reduction in calcium potential. At its most effective, when the peptide is combined with the fragment from β-amyloid, then this enhanced calcium entry followed by the triggering of intracellular control mechanisms, is seen as a spontaneous oscillation. It has already been shown that recombinant AChE, acting in a non-classical fashion, can enhance calcium entry into these neurons via a modulatory action on the NMDA receptor. These results suggest that the peptide derived from AChE, specified in the invention, could be responsible for this effect.

(ii) Behavioural Studies.

In these experiments, rats were chronically implanted with a cannula in one substantia nigra and left to recover. After a period of about 3 days, they were infused with either a saline control solution, or a solution containing the 14-mer AChE peptide of the invention at a dose of 1μl of 10^{-5}M. After a single infusion, they were challenged daily with a systemic application of amphetamine for the subsequent 10 days. Although the control group (n=6) showed no significant effects, the group receiving the peptide (n=8) gradually started to display contraversive rotation, which reached a maximum after 7 days post infusion and remained consistent for

the remaining 3 days tested.

These results suggest that the peptide-mediated calcium entry observed in (i) could be setting in train long-latency, long-term intracellular events that result in a sustained elevation of the activity of neurons in the treated substantia nigra. This enhanced, unilateral activation is manifest as contraversive circling behaviour.

CLAIMS

1. A peptide containing at least 6 amino acid residues and having at least 70% homology with part or all of the sequence

AEFHRPSSYMVHWK.

2. A peptide comprising or consisting of the sequence YMVH or MVHW or VHWK and having at least 70% homology with part or all of the sequence

AEFHRPSSYMVHWK.

3 A mixture of the peptide of claim 1 or claim 2 with another peptide having at least 4 amino acid residues and having at least 70% homology with the β-amyloid precursor sequence

DAEFRHDSGYEVHHQK.

4. A probe consisting of the peptide of claim 1 or claim 2 or the mixture of claim 3, labelled with a signal moiety, or immobilised on a support.

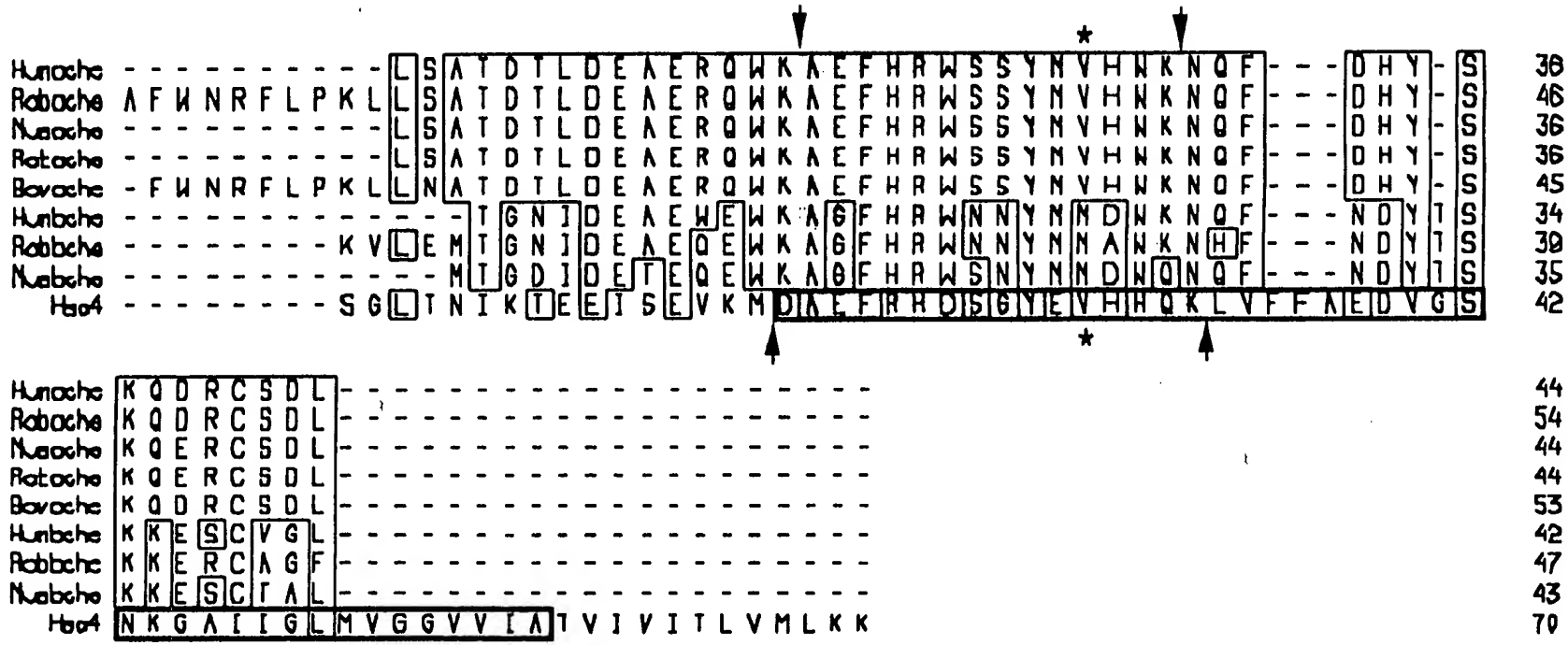
5. A compound which inhibits a biological activity of the peptide of claim 1 or claim 2 or the mixture of claim 3.

6. A compound as claimed in claim 5, which is capable of crossing the blood-brain barrier.

7. A method of preparing a composition for treatment of a disorder of the central nervous system or stroke or cancer, which method comprises bringing a compound according to claim 5 or claim 6 into a form for human administration.

8. A method of preparing a composition for controlling cytoplasmic calcium ion concentration *in vivo*, which method comprises bringing a compound according to claim 5 or claim 6 into a form for human administration.

Multiple sequence alignment of five AChE sequences, three BuChE sequences and the human amyloid precursor polypeptide at the region of interest



The polypeptide sequences are Humache = Human AChE; Rabache = rabbit AChE; Musache = mouse AChE; Ratache = rat AChE; Bovache = bovine AChE; Humbche = human BuChE; Rabbche = rabbit BuChE; Musbche = mouse BuChE; hsa4 = human A4 amyloid precursor polypeptide
Residues in red are conserved across all sequences; residues in green are shared by all AChEs and A4, but by none of the BuChEs

The synthetic peptides are denoted by blue shading and the amyloid peptide 1-42 is shown as a dark blue box
The asterisk marks the conserved valine critical for the dihedral angle between the N terminal alpha helix and the next domain of the 1-42 amyloid peptide